CHEMICAL CARCINOGENESIS AND CYCLIC AMP

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The apparent rigorous control which is exercised over cell division in each organ led to the assumption that there exist substances in tissues which regulate cell division. This hypothesis usually has suggested that such compounds should act as inhibitors and that cells will divide at a maximal rate in the absence of the inhibitor. A search for such substances led us to investigate adenosine 3',5'-monophosphate (cyclic AMP) as a potential regulator of cell division.

Cyclic AMP was found to inhibit cell division in tissue culture and to do so in a unique manner. There is no apparent toxicity, such as detachment from the glass, even at concentrations of up to 3 mM. Removal of the cyclic AMP-containing medium permits the cells to divide again in a normal manner (1). The variety of means available for elevating the levels of cyclic AMP by means of adenylate cyclase and lowering the levels by phosphodiesterase permit cyclic AMP the requited flexibility to carry out this possible role as a regulator of cell division.

Considerable evidence has accumulated which suggests a correlation between the cellular level of cyclic AMP and the rate of cell division (2-5). A further development has been the comparison of the levels of cyclic AMP, or one or more of the enzymes of the cyclic AMP system in normal, transformed, or malignant cells (6-14). At present, most evidence indicates that the rapidly dividing cell has lower levels of cyclic AMP, and for this reason it is concluded that the transformed and malignant cell may have defects in the cyclic AMP system.

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Although transformation of cells in vitro results in a number of interesting cellular changes, none of these changes seem to be uniquely associated with tumorigenicity or neoplasia (15, 16). For this reason, cell lines of known tumorigenicity and lineage were sought. An appropriate cell system for studying the changes in the cyclic AMP system following chemical carcinogenesis is that of Freeman et al (17). A normal rat embryo cell line, F111, free of murine viruses, was transformed in vitro by a variety of chemical carcinogens and by the Rauscher Leukemia Virus. The resultant cell lines produce tumors in the neonatal rat. In addition, the normal rat embryo cell of origin is available for comparison with the tumorigenic cell lines (Figure 1).

The aim of this study is to assay the cyclic AMP system in normal and carcinogenic treated cells. In addition, investigations of the response of the tumor cell in vitro to cyclic AMP and cyclic AMP elevating agents may provide a rational basis for an approach to inhibiting the growth of the tumors produced in vivo by these cell lines.

MATERIALS AND METHODS

Cell Culture

The rat embryo cell lines (F111 p. 32-34, F1706 p. 118-122, F1849 p. 59-62, and F2412 p. 36-37) were seeded in 20 X 100 mm plastic culture dishes (Falcon) at a cell density of 5000/cm^2 in Dulbecco's Modified Eagle's Medium with glutamine (Grand Island Biological) with 10% fetal calf serum. To the medium was also added sodium bicarbonate (13.5 g/1), amphotercin B (2 mg/1), penicillin (50,000 units/1), and streptomycin (500 mg/1). The cells were incubated at 37° in an atmosphere of 5% CO_2 and 95% air. The cells were removed from the dishes with 0.25% trypsin. All cells were counted on a Model Fn Coulter Counter.

The inhibition studies were conducted in 60 mm petri dishes containing 5 ml. of Dulbecco's Modified Eagle's Medium with 10% fetal calf serum. The percent inhibition=

$$\frac{\text{Cell count (control)} - \text{cell count (treated)}}{\text{Cell count (control)}} \times 100$$

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Adenylate Cyclase

After removing the culture medium, the cells were washed with 10 ml. of 0.85% saline. For values obtained at 24 hours, 48 hours, and 96 hours of incubation, the cells were removed by scraping. For the zero time value the cells were removed by trypsinization and 3 ml. of fetal calf serum added to inhibit the action of trypsin. After removal, the cells were centrifuged and the cells were prepared as described by Makman with the exception that 5 mM dithioerythritol was added to the buffer (13, 17).

The incubation conditions were modified from those described by Makman and Krishna and were determined to give linear rates of activity for 20 min. at 30° C (13, 17, 18). Incubations were carried out in 10 X 75 mm test tubes containing: 1.0 mM ^3H ATP, 4.5 mM $MgCl_2$, 2.0 mM cyclic AMP, 2.5 mM phosphoenolpyruvate, 3.0 mM theophylline, 1.25 mg/ml pyruvate kinase, 45 mM Tris-HCl pH 7.6, and either 8 mM NaF or the Tris-Mg buffers in a final volume of 200 µl. The reactions were started by addition of cell homogenates to give final concentrations ranging from 1.0 to 2.0 mg. protein per milliliter of assay. Incubations were carried out at 30° C and were terminated by the addition of 0.2 milliliter of 50 mM Tris/HCl, pH 7.6, 5 mM cyclic AMP, and 3 mM ATP. The reaction mixtures were heated for two minutes in a boiling water bath after which 0.6 ml. of distilled water was added per tube. The cyclic AMP was isolated and measured as described by Krishna (18).

Cyclic AMP Assay

The medium was removed from the cells and drained as completely as possible. To each dish of cells was added 5 ml. of 0.5 N perchloric acid in 25% ethanol (v/v) containing tracer cyclic AMP for estimation of recovery. The precipitated cell sheet was scraped, washed, and centrifuged. The supernatant was neutralized with saturated KOH. After removal of the precipitate, the supernatant was chromatographed by the method of Brooker (19). The cyclic AMP assay was used as described by Gilman (20). A sample from each series was treated with phosphodiesterase

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as described by Otten et al (4).

Cell Volume Determinations

For cell volume determinations, the growth media was removed from exponentially growing monolayer cultures and replaced with fresh growth media containing the test reagents. Following incubation, the cells were washed with 2 ml. Dulbecco's Tris buffer (0.025 M Tris, 0.1 M KH_2PO_4, and 0.25 M saline, pH 6.8-7.0) then incubated with 1 ml. of 0.25% trypsin for 5 minutes at 37°. The detached cells from each plate were placed in 100 ml. of Dulbecco's Modified Eagle's Medium, pH 7.2, (containing 0.04 M Tris buffer) supplemented with 10% fetal calf serum, penicillin (500 units/ml.), and streptomycin (500 g/ml.) and the cell volume measured with a Model B Coulter Counter.

RESULTS

In Figure 1 is a summary of some of the characteristics of four of the rat embryo cell lines. The most rapidly dividing cell is the F2412, which was transformed by 3-methylcholanthrene. The cell volume of the two transformed cell lines (F2412 and F1849) is very similar, and the largest of the four is the normal cell line, F111. The F2412 and F1849 produce tumors in the neonatal rat, whereas the F111 and F1706 do not.

The basal and fluoride stimulated levels of adenylate cyclase of each of the four cell lines is compared in Table 1. The only remarkable difference in the values is the elevation of the basal level of the F2412 at 120 hrs. The elevation of the basal values at zero time must be due to the effect of trypsinization or the addition of fresh serum, which is added to inhibit the trypsin, since the zero time values are obtained by trypsinization of 120-hour cultures. Since the fluoride affects ATPase and adenylate cyclase, which are membrane bound enzymes, presumably the chemical carcinogen has altered the membrane of the F2412 cells. Similar elevations are seen in the zero time values for the fluoride stimulated levels of F111, but not F2412. The lowest values for basal and fluoride stimulated levels of adenylate cyclase are found at 18 hours and 48 hours during which time the

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cells are multiplying most rapidly.

Similarly, the highest values obtained for cyclic AMP (Table 2) in the cell lines were found between 0 and 8 hours after trypsinization of the cells. In general, all of the cyclic AMP values decrease after 8 hours reaching their lowest levels at 168 hours. The F2412 and F1849 cells have the lowest levels of cyclic AMP and this is particularly apparent during logarithmic growth. From comparison of the adenylate cyclase and cyclic AMP levels, it appears that a correlation exists between basal levels of adenylate cyclase and cyclic AMP levels except for the 120-hour value of F2412.

The effects of adding exogenous cyclic AMP or dibutyryl cyclic AMP is generally found to retard the rate of division of cells. Because the cells metabolize the added cyclic AMP to 5' AMP, which may also be inhibitory, inhibition is a consequence of both cyclic AMP and 5' AMP. For this reason, it is best to assay the effect of cyclic AMP on cells at several cell densities. This relation between cell density and cyclic AMP inhibition has been demonstrated by van Wijk, Wicks, and Clay (21). The effects of exogenous cyclic AMP on each of the cell lines is shown in Table 3. The tumor cell line F1849 was not inhibited by cyclic AMP and F2412 was only slightly inhibited, whereas the normal cell lines were markedly inhibited. An explanation for this difference was presented by a previous investigation with WI-38 cells which were also found to be resistant to inhibition by cyclic AMP. This resistance is related to a decreased ability to transport cyclic AMP (22, 23). To test this possibility, each of the four cell lines were incubated in 0.3 mM cyclic AMP for 24 hours and the percent inhibition and cyclic AMP level in each of the cells measured. The results in Table 4 show that there is a correlation between the level of cyclic AMP and the inhibition observed. The tumor cells have considerably lower levels of cyclic AMP than the normals, which suggests a possible mechanism for their resistance to exogenous cyclic AMP. This may be due to a decreased transport of cyclic AMP, or may also result from more rapid destruction by phosphodiesterase.

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An attempt to inhibit these tumors in vivo by injection of cyclic AMP would appear futile. However, other agents such as theophylline, the prostaglandins, and dibutyryl cyclic AMP may inhibit the cells. An investigation of the inhibitory effects of these agents is shown in Table 5. Although cyclic AMP does not inhibit the carcinogen or virus transformed cells, theophylline is more inhibitory to these two tumor cells than it is to either of the normal cells. Although dibutyryl cyclic AMP inhibits all four of the cell lines, the addition of equimolar butyrate to the cells is equally inhibitory which probably indicates that the inhibition by dibutyryl cyclic AMP is actually due to the butyrate. The prostaglandin E_1 is more inhibitory to the normal cells than to the tumor cells. The results suggest that theophylline might be more effective than PGE_1 or cyclic AMP in inhibiting the growth of the tumor cells in vivo.

DISCUSSION

The basal adenylate cyclase levels of the 3-methylcholanthrene transformed cell line (F2412) and its cell of origin (F111) are similar except when the cells reach confluency. At confluency the basal level of adenylate cyclase in the F2412 cell line is approximately three times as great as the normal cell. The significance of this increase is difficult to interpret, as the F111 cell line shows a sharp decrease in growth at confluency whereas the F2412 continues to grow and is not density inhibited. To obtain zero time values, cells from confluent cultures were trypsinized. Since this is the only difference in treatment between the 120-hour values and the zero time values for adenylate cyclase, the marked increase in the basal values for this time period must be due to the trypsinization procedure. Although the normal cells show a 7-fold (basal) and 2-fold (fluoride) increase following the trypsinization, the tumor cell (3-MC) is increased only 2-fold in the basal level and in the fluoride level not at all. Effects of trypsin on cell division, cyclic AMP levels, and on membrane phenomenon have been reported. The elevation of adenylate cyclase by trypsinization of normal cells, with slight effect on tumor cells, may also be related to the above phenomena.

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Some correlation is seen between the levels of adenylate cyclase and cyclic AMP in the cell; for example, the highest cyclic AMP and adenylate cyclase values are found after trypsinization. The cyclic AMP levels in the cells decrease from this point and reach their lowest levels at confluency. The lack of correlation between cyclic AMP and adenylate cyclase values, especially in the F2412 cell line, after the first 48 hours may be due to an increase in phosphodiesterase which has been shown to be an inducible enzyme (24, 25). A rise in phosphodiesterase may lower the cyclic AMP levels in spite of the increase in adenylate cyclase. Although the values for cyclic AMP in the four rat embryo cell lines are similar to those reported by others (3), they are at least 10-fold higher than those that we have found for the 6C3HED, L5178Y, and L1210 tumor cells in vivo and nearly 100 times greater than normal mouse liver and spleen (26). The explanation for these considerable differences is not presently clear. Neither of the tumor cells are inhibited by exogenous cyclic AMP, whereas the normal cells are greatly inhibited. A possible explanation for this difference is the observation of lower cellular levels of cyclic AMP found in the tumor cells after incubation in cyclic AMP. This may be due to a reduced ability of cyclic AMP to be transported by the tumor cells or to higher phosphodiesterase levels in the tumor cells. The inability to effectively transport cyclic AMP may represent the loss of an important control mechanism. Cells at high density, as in tissues, may regulate the rate of cell division of each other by inter and intracellular transport of cyclic AMP. Loss of this relationship may permit the tumor cell to grow independently of other cells in the organ without the restraint of cyclic AMP.

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SUMMARY

A normal rat embryo cell line was transformed *in vitro* by 3-methylcholanthrene to a cell which produces tumors in the Fischer rat. The adenylate cyclase, cyclic AMP level during growth in tissue culture and response to exogenous cyclic AMP were investigated. For comparison a high passage rat embryo cell line and a cell line transformed by RLV, arising from the same original cell line were also examined. The carcinogenic transformation results in a cell of smaller cell volume which divides more rapidly. Treatment with trypsin increases the basal level of adenylate cyclase in the normal cell considerably, but alters the adenylate cyclase of the malignant cell only slightly.

The inability of cyclic AMP or dibutyryl cyclic AMP to inhibit the tumor cells is believed to be due to a decreased ability to transport cyclic AMP. This may represent the loss of an important control mechanism in which cells in organized structures control the rate of division of each other.

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Table 1

Adenylate Cyclase of Rat Embryo Cell Lines

Basal Level

Time (hours)	F111	F1706	F1849	F2412
0	47.7	13.2	10.2	45.6
18	2.6	3.9	1.3	3.2
48	7.5	7.8	2.3	5.9
120	7.7	8.7	3.4	23.6

Fluoride-Stimulated Level

Time (hours)	F111	F1706	F1849	F2412
0	124.0	43.0	102.0	62.0
18	5.4	11.8	10.3	5.9
48	48.0	29.0	17.0	11.0
120	67.0	59.0	20.0	68.0

Values pmoles cyclic AMP formed/minute/mg. of protein at 30°. Linear rates were obtained for 20 minutes. The 15 minute value was used for the calculations. Values for 0 time obtained by trypsinization of confluent cultures. Logarithmic growth obtained at 18 and 48 hours. Cells were confluent at 120 hours. Cells were plated at a density of $50/mm^2$.

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Table 2

Cyclic AMP Level of Rat Embryo Cell Lines During Growth

Hours	F111	F1706	F1849	F2412
0	60	200	336	97
8	102	120	67	43
24	46	84	29	19
48	50	68	18	15
120	34	9	16	16
168	15	3	10	4

Cells were seeded at $50/mm^2$. Values are pmoles/10^6 cells. Average of five determinations. Logarithmic growth occurs at 24 and 48 hours and cells are confluent at 120 hours. To convert pmoles/10^6 cells to pmoles/10^{-1} mg. multiply values by: F111 = 0.42, F1706 = 0.39, F1849 = 0.48, and F2412 = 0.76.

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Table 3

Cyclic AMP Inhibition of Rat Embryo Cell Lines

	F2412			F1849	
mM	A	B	mM	A	B
0.3	0	10	0.3	0	0
0.7	18	26	0.7	0	0
1.0	14	28	1.0	0	0

	F1706			F111	
mM	A	B	mM	A	B
0.3	23	48	0.3	14	35
0.7	46	65	0.7	59	65
1.0	57	74	1.0	60	69

Cell density for A = 54 cells/mm^2; B = 27 cells/mm^2.
Percent inhibition 72 hours after plating of cells.

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Table 4

Level of Cyclic AMP in Rat Embryo Cells
After Incubation in Exogenous Cyclic AMP

	Cyclic AMP/10^6 Cells	% Inhibition
F1706	7546	32
F111	5038	20
F1849	3807	0
F2412	2100	0

Cells were plated at a density of 50 cells/mm^2. 0.3 mM cyclic AMP added to each experimental group at the time of plating. Cells were washed six times with cold saline before the addition of perchloric acid. Cyclic AMP levels and percent inhibition determined at 24 hours. Values are an average of four determinations.

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Table 5

Response of Rat Embryo Cell Lines to Theophylline, Dibutyryl Cyclic AMP, Cyclic AMP, and PGE_1

Inhibitor	F2412	F1849	F1706	F111
Theophylline, 1 mM	84	75	26	40
Dibutyryl Cyclic AMP, 1 mM	39	53	54	65
Butyrate, 1 mM	39	38	48	68
Cyclic AMP, 1 mM	10	0	65	62
PGE_1 25 µg/ml.	13	0	20	44

Percent inhibition determined after 72 hours incubation. Cells were plated at a density of 50 cells/mm^2.

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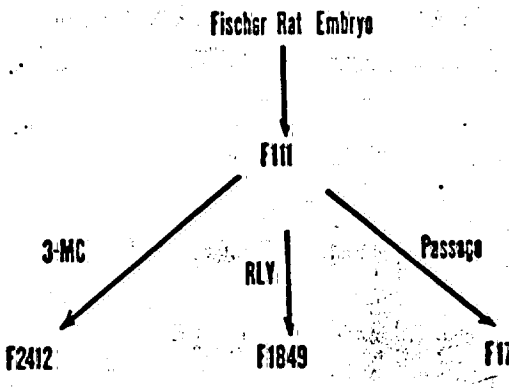
FIGURE 1. ORIGIN AND CHARACTERISTICS OF RAT EMBRYO CELL LINES



	Population Doubling Time	Passage Number	Cell Volume μ^3	Tumorigenicity Neonatal Rat
F111	23	32-34	3200	No
F1706	20	118-122	2100	No
F2412	15	36-37	2600	Yes
F1849	23	59-62	2500	Yes

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